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Characterization and measurement of dehydroepiandrosterone sulfate in rat brain

(adrenal androgen/stress/Δ^5-3β-hydroxysteroids/testosterone)

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ABSTRACT **Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, I) sulfate (Ia) has been characterized in the anterior and the posterior parts of the brain of adult male rats. Its level (1.58 ± 0.14 and 4.89 ± 1.06 ng/g, mean ± SD, in anterior and posterior brain, respectively) largely exceeded that of I in brain (0.42 ± 0.10 and 0.12 ± 0.03 ng/g in anterior and posterior brain, respectively) and of Ia in plasma (0.26 ± 0.13 ng/ml). Brain Ia level did not seem to depend on adrenal secretion; it was unchanged after administration of corticotropin or dexamethasone for 3 days, and no meaningful change occurred in brain 15 days after adrenalectomy plus orchiectomy, compared with sham-operated controls. In contrast, stress conditions prevailing 2 days after adrenalectomy plus orchiectomy or after the corresponding sham operation resulted in a significantly increased concentration of Ia in the brain. Changes of Ia level in brain occurred irrespective of changes in corresponding plasma samples. It is proposed that Ia formation or accumulation (or both) in the rat brain depends on *in situ* mechanisms unrelated to the peripheral endocrine gland system.**

Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, I) sulfate (Ia) is below detection limit in the plasma of most adult mammals (1); the exceptions are man and the highest nonhuman primates (1–3). It is a major secretory product of human adrenals (4–7), and its concentration in adult plasma is larger than that of any other steroid. Although Ia is the main precursor of placental estrogens (8–10) and is occasionally converted into active androgens in peripheral tissues (11, 12), no obvious biological function has been attributed to it in normal individuals (13).

This report shows that in adult rat brain, Ia is present at a concentration up to 20 times that in plasma. Ia persists in the brain after adrenalectomy and castration of male rats, suggesting that it may be synthesized or selectively retained in the central nervous system or both.

MATERIALS AND METHODS

Animals. Male and female Sprague–Dawley rats (OFA strain, Iffa Credo, L'Arbresle, France) were kept at 18°C under a 12 hr/12 hr lighting schedule (0800–2000). As indicated, they were castrated 24 hr or castrated and adrenalectomized 48 hr or 15 days before sacrifice. Sham-operated controls were submitted to anesthesia, incision, and organ manipulation. Animals were fed ad lib and adrenalectomized animals received isotonic saline as drinking water. They were killed at the age of 77 ± 2 days. Decapitation was performed between 1000 and 1100. Blood was collected in heparinized flasks and centrifuged at 0–4°C. Organs were quickly removed and processed at 0–4°C. Adrenals, liver, spleen, and testes were taken as such. The brain was divided in two parts: (*i*) a posterior part containing the cerebellum, the pons, and the medulla oblongata (mean weight 0.37 ± 0.05 g) and (*ii*) an anterior part containing the cortex cerebri and the mesencephalon and excluding the bulbus olfactorius, the hypothalamus, and the thalamus (mean weight 1.12 ± 0.09 g).

Chemicals. The purity of [1,2,6,7-^{3}H]I (specific activity 79 Ci/mmol, 1 Ci = 3.7 × 10^{10} becquerels; Amersham) and [7α-^{3}H]IA ammonium salt (specific activity 24 Ci/mmol, New England Nuclear) was checked by thin-layer chromatography on silica gel (GF 254, Merck), using chloroform/ethyl acetate (80:20) for I and ethyl acetate/methanol/ammonia (75:25:2) for Ia. Reference I and Ia were gifts of Roussel Uclaf, Romainville, France. All solvents were of analytical grade and were redistilled before use.

Measurements of I and Ia. The technique of Ruokonen *et al.* (14) was used with minor modifications. Briefly, [^{3}H]Ia [3000 dpm, (1 dpm = 16.7 mBq) 16 pg] and [^{3}H]I (3000 dpm, 5 pg) was added to 2 ml of plasma or <1 g of tissue, and then 5 ml of acetone/ethanol (1:1) was added. Tissues were homogenized in acetone/ethanol (1:1) with a Teflon/glass homogenizer and sonicated with a Branson J1 sonifier equipped with a minitip at a 100-W setting for 10 sec. The suspensions were kept at 39°C overnight and centrifuged at 1000 × *g* for 10 min. The supernatant was saved, and the pellet was extracted with 4 ml of methanol/chloroform (1:1) with continuous shaking at room temperature for 30 min. The extract was centrifuged, and the two supernatants were combined and taken to dryness. The residue was dissolved in 4 ml of methanol/chloroform (1:1)/10 mM NaCl and deposited on a Sephadex LH-20 column (10 × 445 mm) equilibrated and developed in the same solvent system (15). The first 50 ml to run off contained unconjugated I, Ia was eluted in the next 75 ml. It was completely separated from free I and from I conjugated to fatty acids (16) or glucuronic acid. Ia-containing eluates were solvolyzed in ethyl acetate at 37°C for 12–16 hr to quantitatively generate free I (17). The extracts were washed with water, filtered through Na_2SO_4, and dried. They were chromatographed on Celite 535 columns, according to Brenner *et al.* (18).

I was measured by a specific radioimmunoassay (19). The only crossreactions were with 5-androsten-3β,17β-diol (3.4%) and 4-androsten-3,17-dione (0.4%), which were separated from I during the column partition step, and with epiandrosterone (1.6%). After oxidation, which transforms I into 4-androsten-3,7,17-trione (20), no immunoreactive steroid was left in the I-containing fraction. The recovery of I added as a tracer was 50–60%, and the recovery of Ia was 30–45%.

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Abbreviation: TMS, trimethylsilyl.

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Measurements were made in triplicate on samples containing ≈50 pg of I. The sensitivity of the assay was such that >30 pg of I or >40 pg of Ia could be determined in 1 ml of plasma or 1 g of tissue.

Characterization of Ia. Anterior and posterior brain were taken from 14 male rats. Tissues were homogenized in water and, after removal of free steroid and accompanying fatty acid conjugates with dicholoromethane, steroid conjugates were extracted with ethyl acetate, pH 1/20% NaCl (wt/vol) (17). After solvolysis, a portion was kept for radioimmunoassay and the remainder was processed. The anterior and posterior brain final extracts contained 11 and 12 ng of I, respectively (uncorrected for losses). Tracer [^{3}H]I (1.3×10^5 dpm, ≈200 pg) was added to each extract. After removal of solvents under N_2, the residue was dissolved in 0.5 ml of hexane/chloroform, 8:2 (vol/vol) and applied to a column of Lipidex 5000 (0.5 g, 150 × 4 mm) equilibrated in the same solvent (21, 22). The column was washed with 3.5 ml of solvent, and I was eluted with 3 ml of solvent. This fraction was dried under N_2. Trimethylsilyl (TMS) ethers were prepared in trimethylchlorosilane/hexamethyldisilazane/pyridine (1:2:3) at 60°C for 30 min. Reagents were removed under N_2, and samples containing ≈200 pg were dissolved in 50 μl of hexane for gas chromatographic/mass spectrographic analysis. A modified LKB 9000 instrument was used with a 25 m × 0.3 mm open-tubular glass capillary column coated with SE-30 and connected to the ion source via a single-stage adjustable jet separator (23). Temperatures of column, separator, and ion source were 260°C, 260°C, and 310°C, respectively. The energy of bombarding electrons was 22.5 eV. Slits were set to give sufficient resolution and flat-top peaks. Repeated analyses were performed by using a constant magnetic field focusing single *m/z* values of diagnostic importance onto the detector. The TMS ether of 5β-cholestane-3β-ol was added in a known amount, sufficiently large to be detected in the total ion current chromatogram, and thus served as an internal standard to permit quantitative comparison among the different analyses. Samples and reference compound were analyzed under identical conditions and ion current chromatograms were obtained for the following ions: *m/z* 360 (M^+), 304 ($M - 56$) (24), 270 ($M - 90$), 231 ($M - 129$), and 129. Under the conditions used, *m/z* 129 carried ≈20% of the total ionization and was the base peak in the spectrum of authentic I TMS ether. Quantitations were made by comparison of peak heights with those given by known amounts of I TMS ether.

RESULTS

Characterization of Ia. Preliminary identification of Ia was based on the following criteria: (*i*) extraction of steroid conjugates with polar solvent mixtures, (*ii*) chromatography on Sephadex LH-20 and isolation of the fractions containing steroid monosulfates ([^{3}H]Ia was used as internal standard), (*iii*) solvolysis, (*iv*) partition chromatography on Celite columns and isolation of I-containing eluates, and (*v*) radioimmunoassay using a specific antiserum having minimal crossreaction with other steroids.

Definitive identification of the steroid moiety was made by using pooled anterior and posterior brain extracts from 14 male rats. After solvolysis and chromatography on Celite and Lipidex columns, a highly purified fraction was obtained, as judged by the absence of significant peaks in the total ion current chromatogram. As shown in Fig. 1, peaks with a retention time of 6.1 min appeared in the chromatogram of all the diagnostically important *m/z* values. Authentic I TMS ether injected in similar amounts gave the same peaks at the same retention time. The relative intensities of the peaks were the same for the compounds from brain and the reference compound within experimental error (Table 1).

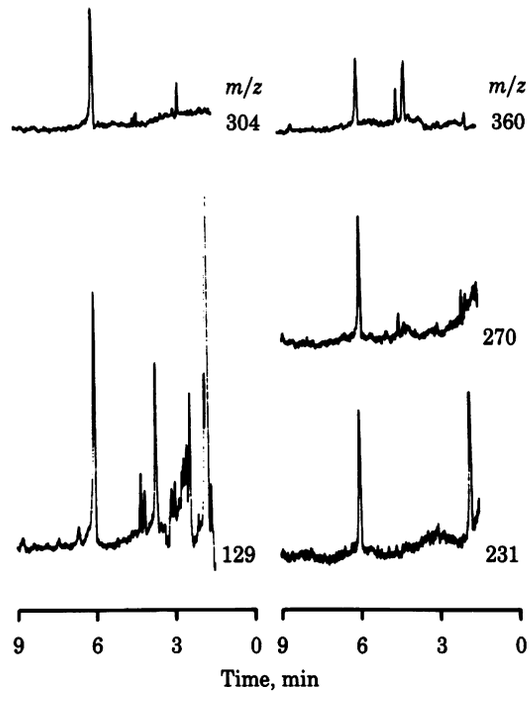

FIG. 1. Single ion current chromatograms obtained in analysis of TMS ether of purified steroid from posterior brain. Major ions given by the I TMS ether (eluted at 6.1 min) were selected. The signal of *m/z* 129 was attenuated four times. Peaks at 6.1 min represent ≈200 pg of steroid.

The amount of steroid was calculated from the heights of the peaks in the *m/z* 360 chromatograms. The sample from anterior brain was found to contain 11 ng and that from posterior brain contained 12 ng of I, in agreement with the values measured by radioimmunoassay.

Based on the retention time of the TMS ether on a glass capillary column, the relative intensities of diagnostically important ions and the quantitative comparison with the radioimmunoassay results, the compounds released by solvolysis from anterior and posterior brain extracts were identified as I.

Ia in Plasma and Organs of Intact Rats. Measurements were performed on single animals except for adrenals (four glands taken from two rats) and plasma (pools of three rats). They were repeated at least three times (Table 2). Low concentrations of Ia were found in plasma of rats, at the limit of sensitivity of the technique. Higher concentrations were found in liver, spleen, testis, and kidney, but they were well below 1 ng/g of tissue. In sharp contrast was the level of Ia in the brain, in particular

Table 1. Relative intensities of diagnostically important mass spectral peaks of I TMS ether and TMS ethers of compounds isolated from brain fractions of male rats

	TMS ether		
m/z	I	Anterior brain	Posterior brain
360	8	7	6
304	11	13	11
270	14	15	12
231	20	20	14
129	100	100	100

I was purified after solvolysis of extracts from 14 male rats. TMS ethers were prepared and processed as described in *Materials and Methods*.

Table 2. **Ia** and **I** in plasma and organs of male rats

	Ia, ng/g	**I**, ng/g
Plasma	0.26 ± 0.13 (9)	0.08 ± 0.02 (4)
Brain		
Anterior	1.58 ± 0.14 (10)	0.42 ± 0.10 (4)
Posterior	4.89 ± 1.06 (11)	0.12 ± 0.03 (4)
Adrenals	2.90 ± 0.30 (4)	ND
Liver	0.43 ± 0.08 (3)	0.04 (1)
Spleen	0.65 ± 0.06 (3)	0.06 (1)
Testes	0.42 ± 0.01 (3)	ND
Kidneys	0.46 ± 0.03 (3)	0.05 (1)

Each extract was obtained from a single animal, except for adrenals (four glands from two rats) and plasma (pools of three rats). Results represent mean ± SD. Values in parentheses are numbers of experiments. ND, not done.

in the posterior brain (4.89 ± 1.06 ng/g), which was even larger than the concentration in adrenals.

Free **I** was close to the detection limit in plasma, posterior brain, liver, spleen, and kidney, but its level was 0.42 ± 0.10 ng/g in anterior brain.

Ia was also measured in two 11-week-old female rats; the values in the plasma (0.29 ng/ml) and in anterior and posterior brain (1.4–1.6 and 2.9–3.0 ng/g, respectively) were of the same order of magnitude as those of males of the same age.

Ia in Plasma and Organs After Endocrine Manipulation of Male Rats. Several experiments were performed to gain insight into the origin of **Ia** in brain.

Injections of long-acting zinc phosphate preparations of corticotropin (ACTH; β1-24 ACTH, CIBA–Geigy) or of dexamethasone for 3 days were not accompanied by clear-cut changes of brain **Ia** (Table 3). Brain **Ia** was also unchanged 1 day after castration. It was slightly but significantly decreased 15 days after sham operation. No obvious change was observed when castrated and orchiectomized males were compared 15 days after operation with sham-operated controls. In contrast, brain **Ia** was significantly increased 2 days after castration and adrenalectomy or corresponding sham operation.

No significant changes of plasma **Ia** were recorded, with the exception of a small but significant increase after corticotropin injection and 2 days after sham operation or adrenalectomy.

DISCUSSION

Our results demonstrate the presence of **Ia** in brain in amounts exceeding those in plasma and in organs such as liver, kidney, spleen, testis, and even adrenal. **Ia** did not disappear or decrease in brain 15 days after orchiectomy, adrenalectomy, or both or after inhibition of adrenal secretion by dexamethasone. It did not increase after injection of long-acting corticotropin. The results observed 2 days after orchiectomy plus adrenalectomy and the corresponding sham operations were remarkable. In both cases, there was an increase—to 4–6 ng in anterior brain and 9–13 ng/g in posterior brain—irrespective of essentially insignificant variations in corresponding plasma samples.

Our results could be explained by a large accumulation of **Ia** in the brain combined with very slow turnover. Indeed, contrary to previous studies, which found a lack of 17α-hydroxylase activity in rat adrenals, the present methodology can detect a sizable amount of **Ia**, which suggests that biosynthesis of C_{19} steroids may take place, although admittedly at low rate, in the rat adrenals. The significant increase of plasma **Ia** after corticotropin treatment and the decrease after dexamethasone treatment strongly suggest, but do not prove, that **Ia** is secreted by adrenals. Therefore, it cannot be formally excluded that brain **Ia** originates from plasma. Indeed, another steroid hormone sulfate, testosterone sulfate, accumulates in the rat brain (2.3 ng/g in posterior brain). However, **Ia** did not disappear from brain after endocrine ablation, in contrast to testosterone sulfate (<0.03 ng/g in posterior brain 1 day after orchiectomy). Moreover, the concentration of free **I** was much lower than that of testosterone in plasma (2.8 ng/ml) and brain (1.5 ng/g) (25).

Our results lead us to consider the *in situ* formation of **Ia** from biosynthetic precursors. Along this line, we have preliminary evidence for the presence of significant amounts of pregnenolone (61 ± 7 and 26 ± 8 ng/g in anterior and posterior brain, respectively) and its sulfate (6.4 ± 0.5 and 6.7 ± 0.8 ng/g) in the brain of adult male rats. The occurrence of cholesterol sulfate (15 μg/g of dry weight) has been reported previously (26). Several steroid metabolism enzymes have been described in brain, in particular hydroxysteroid dehydrogenases (27–29), steroid-5α-reductase (30–32), aromatase (33), and steroid hydroxylases (28, 34, 35). Steroid sulfokinase activity has been demonstrated in monkey and human brain (28) and steroid sulfatase has been found in rat (36) and human (37) brain. However, *de novo* synthesis of C_{19} steroids from C_{27} or C_{21} precursors has not yet been described in the brain, though the presence of adrenodoxin-like ferredoxin and cytochrome *P*-450 has recently been reported in brain mitochondria (38). The transient increase of brain **Ia** that occurs after the stress produced by adrenalectomy or the corresponding sham operation could be explained by the conversion in the brain of precursors of the Δ^5-

Table 3. **Ia** in plasma and brain of male rats subjected to endocrine manipulations

		Sham operated		ORX	ORX + ADX		ACTH	DXM	EtOH
	Intact	2 days	15 days	(1 day)	2 days	15 days	treated	treated	treated
Plasma	0.26 ± 0.13 (9)	0.59* ± 0.08 (6)	0.28 ± 0.09 (5)	0.21 ± 0.02 (2)	0.40* ± 0.23 (6)	0.36 ± 0.07 (6)	0.50* ± 0.09 (2)	0.14 ± 0.07 (2)	0.56 (1)
Brain									
Anterior	1.58 ± 0.14 (10)	5.77* ± 1.66 (6)	1.19* ± 0.27 (6)	1.45 ± 0.07 (2)	4.35 ± 1.33 (7)	1.63† ± 0.38 (7)	1.35 ± 0.36 (2)	2.10 ± 1.27 (2)	1.9 (1)
Posterior	4.89 ± 1.06 (11)	13.19 ± 2.24 (5)	2.98 ± 0.68 (5)	3.15 ± 0.78 (2)	8.99 ± 1.50 (7)	1.92† ± 0.42 (7)	4.00 ± 1.13 (2)	4.45 ± 1.06 (2)	4.6 (1)

Results are expressed in ng/ml or ng/g (mean ± SD). Each measurement was performed on a single animal; numbers of animals are given in parentheses. Sham-adrenalectomized and orchiectomized rats were sacrificed 2 or 15 days after operation, orchiectomized rats were sacrificed the day after operation, and adrenalectomized and orchiectomized rats were sacrificed 2 or 15 days after operation. Corticotropin (0.5 mg/0.25 ml of solvent) was injected subcutaneously on 2 consecutive mornings and rats were sacrificed on the morning of the 3rd day. Vehicle-injected controls did not differ from intact rats. Dexamethasone (1 mg/0.25 ml of absolute ethanol) was injected subcutaneously on 3 consecutive mornings, and rats were sacrificed 2 hr after the last injection. Ethanol (0.25 ml) was injected to control rats. ORX, orchiectomized; ADX, adrenalectomized; ACTH, corticotropin; DMX, dexamethasone.

* Significantly different from value for intact rats at 5% level.

† Significantly different from sham-operated controls at 5% level.

3β-hydroxysteroid series along a biosynthetic pathway of "neurosteroids."

We have recently observed that **Ia** is present in the brain of mice and that, compared with unaffected siblings, **Ia** was absent or very much decreased in posterior brain of two myelin-deficient mice strains (quaking and jimpy) obtained from N. Baumann and F. Lachapelle (39, 40). Conversely, free **I** was increased in the brain of affected mice. These results support the suggestion that specific mechanisms are responsible for the accumulation of **Ia**.

In conclusion, we have identified **Ia** in the brain of adult rats, and preliminary results suggest its formation or accumulation (or both) *in situ*. The precursors and pathways involved deserve further investigation.

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